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Recovery of Avian Metapneumovirus Subgroup C from cDNA: Cross-Recognition of Avian and Human Metapneumovirus Support Proteins

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Avian metapneumovirus (AMPV) causes an acute respiratory disease in turkeys and is associated with "swollen head syndrome" in chickens, contributing to significant economic losses for the U.S. poultry industry. With a long-term goal of developing a better vaccine for controlling AMPV in the United States, we established a reverse genetics system to produce infectious AMPV of subgroup C entirely from cDNA. A cDNA clone encoding the entire 14,150-nucleotide genome of AMPV subgroup C strain Colorado (AMPV/CO) was generated by assembling five cDNA fragments between the T7 RNA polymerase promoter and the autocatalytic hepatitis delta virus ribozyme of a transcription plasmid, pBR 322. Transfection of this plasmid, along with the expression plasmids encoding the N, P, M2-1, and L proteins of AMPV/CO, into cells stably expressing T7 RNA polymerase resulted in the recovery of infectious AMPV/CO. Characterization of the recombinant AMPV/CO showed that its growth properties in tissue culture were similar to those of the parental virus. The potential of AMPV/CO to serve as a viral vector was also assessed by generating another recombinant virus, rAMPV/CO-GFP, that expressed the enhanced green fluorescent protein (GFP) as a foreign protein. Interestingly, GFP-expressing AMPV and GFP-expressing human metapneumovirus (HMPV) could be recovered using the support plasmids of either virus, denoting that the genome promoters are conserved between the two metapneumoviruses and can be cross-recognized by the polymerase complex proteins of either virus. These results indicate a close functional relationship between AMPV/CO and HMPV.

Avian metapneumovirus (AMPV), previously known as turkey rhinotracheitis virus or avian pneumovirus, causes an acute respiratory disease in turkeys and is also associated with "swollen head syndrome" in chickens (10, 11, 37, 39). The virus was first isolated in South Africa in 1978 and subsequently in other parts of the world (reviewed in reference 25). AMPV was first isolated in the United States in 1996 in Colorado from commercial turkeys showing clinical signs of rhinotracheitis (14, 20). Subsequently, AMPV outbreaks were reported in Minnesota, where the disease has emerged as a major economic problem for turkey farmers. Recent seroprevalence studies have indicated that the virus has also spread to other states, such as North Dakota, South Dakota, Iowa, and Wisconsin (1).

AMPV is a member of the genus *Metapneumovirus* in the subfamily *Pneumovirinae* of the family *Paramyxoviridae* (29). The genus *Metapneumovirus* contains AMPV and the human metapneumovirus (HMPV). HMPV causes an acute respiratory illness in young children and immunocompromised adults (5, 23, 36, 38). Members of the genus *Metapneumovirus* contain a nonsegmented, single-stranded negative-sense RNA genome with the gene order 3′-leader-N-P-M-F-M2-SH-G-L-trailer-5′ (2, 22, 35, 40). The AMPV isolates that exist worldwide are currently classified into four subgroups, namely, subgroups A, B, C, and D. This classification is based mainly on sequence divergence observed in the attachment glycoprotein and the antigenic differences existing among the AMPV strains. The U.S. strains of AMPV belong to subgroup C, while the strains in other parts of the world, especially the European countries, belong to the other three subgroups. Interestingly, sequence analyses have shown that the U.S. subgroup of AMPV is more closely related to HMPV than to its avian counterparts (16–18, 34, 35, 42).

In addition to turkeys and chickens, AMPV can also affect other species of birds (reviewed in reference 25). AMPV infections are often associated with secondary bacterial infections that increase mortality in affected birds. In the United States, AMPV infections and concomitant bacterial infections result in mortality up to 25% among infected birds, resulting in heavy economic losses for the poultry industry (31). Currently, both live attenuated and killed vaccines are being used in the United States to control AMPV infections in affected birds (26, 32). However, the live attenuated AMPV vaccines may cause disease in vaccinated birds, and the killed vaccine has not been very effective in controlling the disease. Hence, a highly stable and efficacious vaccine is needed to control AMPV infections in the United States.

Reverse genetics systems have proved to be powerful means of engineering "tailored" vaccines against various viruses and also of better understanding the molecular biology and pathogenesis of various negative-sense RNA viruses (13). Among AMPVs, an infectious clone system was recently developed for AMPV subgroup A (AMPV-A) that is prevalent in Europe (24). Unfortunately, this system cannot be used to generate live recombinant vaccines for controlling AMPV infections in the United States because AMPV-A is antigenically distinct

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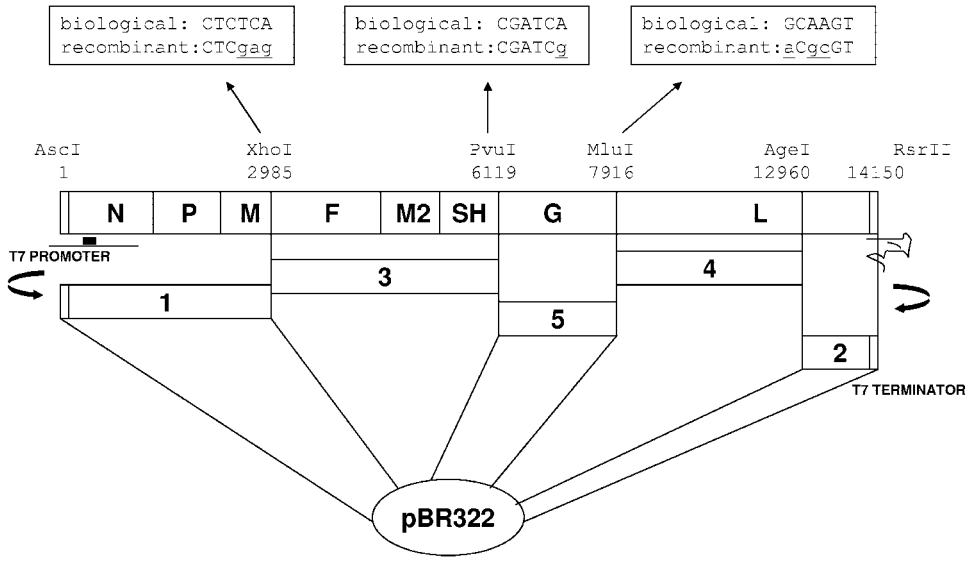

FIG. 1. Generation of full-length AMPV/CO plasmid. Full-length AMPV/CO cDNA was assembled in pBR 322 from five subgenomic cDNA fragments that were generated by high-fidelity RT-PCR. The fragments were inserted in between the T7 RNA polymerase promoter sequence and the hepatitis delta ribozyme autocatalytic sequence, which was followed by the T7 terminator sequence. The names of the restriction enzymes used for the assembly are shown at the top, and the order in which the fragments were assembled is shown on each of them. The XhoI, PvuI, and MluI sites were introduced to facilitate construction and serve as markers (sequence changes shown in lowercase and underlined).

from AMPV subgroup C (AMPV-C) and hence will not be effective against the latter. Furthermore, AMPV-A cannot be used in the United States since it is currently not present here. Hence, to develop a better vaccine for the U.S. subgroup of AMPV, an emerging turkey pathogen, we established a reverse genetics system based on AMPV-C strain Colorado (AMPV/CO), the virus strain whose complete genomic sequence was recently published (17). In addition, we also recovered a recombinant AMPV/CO strain that expresses green fluorescent protein (GFP) as a foreign protein. Both the recombinant viruses showed growth characteristics in cell culture similar to those of the parental virus, except that the GFP-expressing virus grew to a 1-log-lower titer than the parental virus. Interestingly, we were also able to rescue GFP-expressing AMPV/CO and GFP-expressing HMPV using the support plasmids of either virus, denoting that the *cis*-acting sequences of one virus can be recognized by the polymerase complex of the other. These results demonstrate that through the newly established reverse genetics system, it is not only possible to generate genetically engineered AMPV/CO but also to study aspects of molecular biology, pathogenesis, and vaccine development of the closely related HMPV.

MATERIALS AND METHODS

Cells and virus. Vero cells (ATCC CCL-81) were maintained in minimal essential medium (MEM; Invitrogen/GIBCO) supplemented with 10% fetal calf serum. Baby hamster kidney cells that constitutively express T7 RNA polymerase (BSR T7/5 [7]) were a generous gift from Karl-Klaus Conzelmann (Ludwig-Maximilians-University Munich, Munich, Germany). These cells were maintained in Glasgow MEM (Invitrogen/GIBCO) supplemented with 10% fetal calf serum, glutamine, and amino acids under Geneticin (1 mg/ml) selection every second passage. AMPV/CO was obtained from the National Veterinary Services Laboratory (Ames, IA).

Viral RNA extraction. AMPV/CO and the recombinant AMPVs described below were grown in confluent monolayers of Vero cells supplemented with 2% fetal calf serum. Unlike HMPV, exogenous trypsin is not required for growth and/or recovery of AMPV/CO. Virus propagation, purification, and RNA extractions were performed in a manner similar to that described elsewhere (16). Briefly, Vero cells grown to confluence were infected with the parental AMPV/CO or the recovered recombinant virus. Virus was harvested when maximum cytopathic effect (CPE) was evident as extensive syncytia. The infected cells were scraped into the medium and lysed by three cycles of freezing and thawing to release the cell-associated virus. The cell lysate was clarified at $3{,}000 \times g$ for 15 min, and the supernatant was made 10% with respect to polyethylene glycol 8000 (Sigma) and incubated for 3 h at 4°C. Subsequently, the virus was pelleted at $4{,}000 \times g$ for 30 min at 4°C. Viral genomic RNA was extracted from the viral pellet using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, except that an additional extraction with phenol plus chloroform was performed.

Construction of expression plasmids. cDNA fragments bearing the open reading frames (ORFs) of the N, P, M2-1, and L genes of AMPV/CO were generated by reverse transcriptase (RT) PCR. All RT reactions were performed with Superscript II reverse transcriptase (Invitrogen) and gene-specific primers (whose sequences will be available upon request). Plasmid pTM-1 that possesses the encephalomyocarditis virus internal ribosome entry site downstream of the T7 RNA polymerase promoter and uses the translation start codon contained in the NcoI site of the internal ribosome entry site was used for cloning the four genes individually. The N, P, and M2-1 ORFs were cloned individually in pTM-1 between NcoI and BamHI sites. The L gene ORF was cloned between the NcoI and XhoI sites by a two-step cloning procedure using the XmaI site as the third restriction site. The XmaI site was introduced in the L gene ORF without any alteration of amino acid sequence by changing two nucleotides (nucleotide [nt] 9656, A→C; and nt 9659, A→G). The N, P, M2-1, and L ORFs in pTM-1, designated pN, pP, pM2-1, and pL, respectively, were sequenced to their entirety using an ABI 3100 DNA sequencer (Applied Biosystems).

Construction of full-length plasmid. The complete genome of AMPV/CO was cloned into the transcription plasmid pBR322/dr. Plasmid pBR322/dr was a modified form of plasmid pBR322, which contained a 72-nt oligonucleotide linker between the EcoRI and PstI sites and a hepatitis delta viral 84-nt antigenome ribozyme sequence and T7 RNA polymerase transcription termination signal between the RsrII and FseI sites (21). Based on the restriction profile of the complete genome of AMPV/CO, we cloned the AMPV/CO genome into pBR322/dr by using five cDNA fragments (Fig. 1). All cDNA synthesis reactions were carried out using Superscript II reverse transcriptase (Invitrogen) and gene-specific primers. The primers used for RT-PCR of each fragment are listed in Table 1.

Three restriction sites (XhoI, PvuI, and MluI) were artificially introduced

TABLE 1. Oligonucleotide primers used during full-length cDNA synthesis and RT-PCR[a]

cDNA fragment	Orientation	Primer	Cloning order
1	+	5′-GTCA**GGCGCGCC***TAATACGAC TCACTATAGGG*ACGAGAAAAA AACGCATATAAGAC-3′	1
	−	5′-GTCA**ACGCGT**GATCG**CGATCG** TAACTA**CTCGAG**GGTTAAAAA CGAAATTGTTACTGTG-3′	
2	+	5′-TAACC**CTCGAG**TAGTTATTAC CTAGCTTGATATTATTTAG-3′	3
	−	5′-CTTCTC**CGATCG**TTTTTAATCA TTGGATCACCTGTTCTCG-3′	
3	+	5′-AAAA**CGATCG**GAGAAGGAAA AACGGGACAAGTCAACATGG AGGTCAAGGTAGAGAATGTT GGTAAG-3′	5
	−	5′-ATTAACTA**ACGCGT**TTCTAAA CTAACTCCAGCTGTATG-3′	
4	+	5′-GCAT**ACGCGT**TAGTTAATTAA AAAGAAGGACCAAGTTAAAA ATGGATCCAC-3′	4
	−	5′-TGCA**ACCGGT**AGAGCTGAATA CAAAATTG-3′	
5	+	5′-CTCTA**CCGGTT**GCAAAATAAG TGTC-3′	2
	−	5′-AGCT**CGGACCG**cgaggaggtggagatg ccatgccgACCCACGGCAAAAAAA CCGTATTCATCCAA-3′	

[a] The cDNA fragments correspond to the fragments shown in Fig. 1. T7 promoter sequences are marked in italic type, virus-specific sequences are underlined, and restriction sites are marked in bold type. The partial hepatitis delta virus ribozyme sequence (24 nt) overhang is shown in lowercase. +, sense; −, antisense.

during the cloning procedure to help in cloning as well as to serve as markers to confirm the identity of the recovered recombinant virus. The XhoI site was introduced between the M and F genes, in the noncoding region after the M gene termination codon, by altering three nucleotides (nt 2988, T→G; nt 2989, C→A; and nt 2990, A→G). The PvuI site was introduced in the SH-G intergenic region by altering one nucleotide (nt 6124, A→G). The MluI site was introduced between the G and L genes, in the noncoding region after the G gene termination codon, by altering three nucleotides (nt 7916, G→A; nt 7918, A→G; and nt 7919, A→C). None of the artificially introduced marker sites involved amino acid coding sequences. The AgeI restriction site (nt 12960) was a unique site already present in the genome, which was utilized for the cloning process. The five fragments were cloned in the order given in Table 1. After ligation into the plasmid, each fragment was sequenced completely using an ABI 3100 DNA sequencer (Applied Biosystems). The resulting AMPV/CO full-length expression plasmid was termed pAMPV/CO. This plasmid contained three nonviral G residues adjacent to the T7 promoter, at the 5′ end of the antigenome, to enhance promoter efficiency (3).

Construction of full-length plasmid encoding GFP. The antigenomic pAMPV/CO plasmid was modified by the insertion of a transcription cassette containing the ORF for enhanced GFP (Clontech, Inc.). The cassette was introduced as a single fragment at the MluI site created between the G and L genes, in the noncoding region after the G gene termination codon. The transcription cassette contained the MluI recognition sequence, followed by the 14-nt putative G gene end sequence (TAGTTAATTAAAAA, positive sense, AMPV/CO nt 7922 to 7935), followed by a 2-nt intergenic sequence (CC, positive sense), followed by the 16-nt putative N gene start sequence (GGGACAAGTGAAA**ATG**, positive sense, AMPV/CO nt 41 to 56; N-gene ORF initiation codon in bold and underlined), followed by the GFP ORF, followed by the MluI recognition sequence. The fragment was digested with MluI and cloned into pAMPV/CO, resulting in the final construct pAMPV/CO-GFP (Fig. 2). The length of the encoded rAMPV/CO-GFP antigenome, excluding the nonviral sequences, would be 14,905 nt. Construction of pHMPV-GFP has been described elsewhere (3).

Transfection and recovery of infectious recombinant viruses. Transfection experiments were performed in BSR T7/5 cells grown to 90 to 95% confluence in six-well plates. The cells were transfected with 5 μg each of the full-length plasmids (pAMPV/CO and pAMPV/CO-GFP), 2 μg each of pN and pP, and 1 μg each of pM2-1 and pL plasmids in a volume of 100 μl of Opti-MEM per well. Transfection was carried out with Lipofectamine 2000 (Invitrogen), according to the manufacturer's directions. The transfection mixture was removed after 6 h of incubation at 37°C, and the cells were washed and maintained with Glasgow MEM containing 3% fetal bovine serum. Cells transfected with pAMPV/CO-GFP were monitored by fluorescent microscopy (Zeiss) for the expression of GFP. Three to 4 days after transfection, all the media were used for infecting a fresh batch of Vero cells in six-well plates or T-25 flasks and observed for the

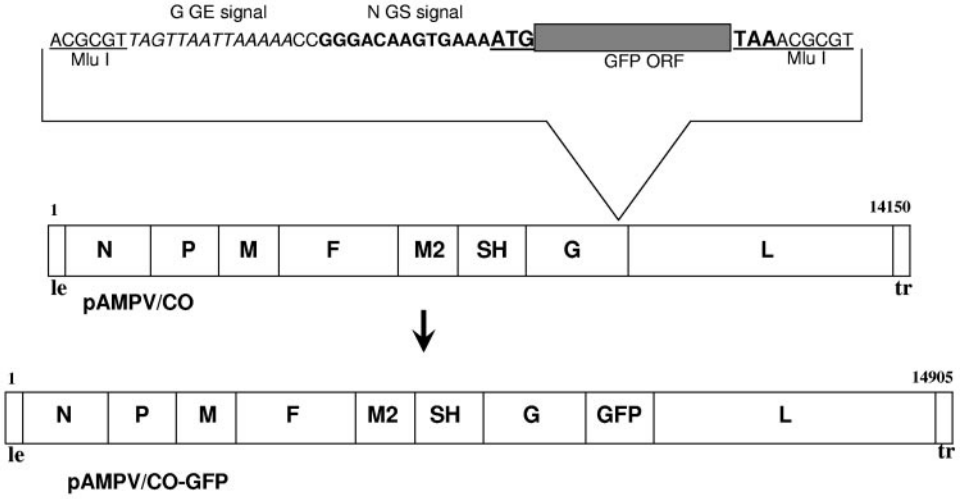

FIG. 2. Construction of plasmid pAMPV/CO-GFP expressing the complete antigenomic RNA of AMPV/CO and GFP as a foreign gene. GFP was inserted in the putative 5′ noncoding region of the G gene as a separate transcription cassette. The cassette contained the GFP ORF (gray rectangle) that was flanked on the upstream side by an MluI site (underlined), the G gene end motif (italicized), an intergenic region comprised of two C residues, and the N gene start motif (bold). The GFP ORF was flanked on the downstream side by an MluI site. The translational initiation and termination codons of the GFP ORF are in bold and underlined. le, leader; tr, trailer.

TABLE 2. Recovery of GFP-expressing metapneumoviruses using heterologous proteins[a]

Plasmid	Support plasmid from:				Virus recovery (%)
	N	P	M2-1	L	
pAMPV/CO-GFP	**HMPV**	AMPV	AMPV	AMPV	10
	AMPV	**HMPV**	AMPV	AMPV	12
	AMPV	AMPV	**HMPV**	AMPV	7
	AMPV	AMPV	AMPV	**HMPV**	28
	HMPV	**HMPV**	**HMPV**	**HMPV**	47
	AMPV	AMPV	AMPV	AMPV	100
pHMPV-GFP	**AMPV**	HMPV	HMPV	HMPV	5
	HMPV	**AMPV**	HMPV	HMPV	6
	HMPV	HMPV	**AMPV**	HMPV	6
	HMPV	HMPV	HMPV	**AMPV**	23
	AMPV	**AMPV**	**AMPV**	**AMPV**	52
	HMPV	HMPV	HMPV	HMPV	100

[a] BSR T7/5 cells were transfected (in duplicate) with the GFP-expressing full-length plasmid of AMPV/CO and HMPV and various combinations of homologous or heterologous (in bold) support plasmids. The actual titers of rAMPV/CO-GFP and rHMPV-GFP recovered using homologous support plasmids were 2.6×10^3 and 3.3×10^3 PFU/ml, respectively. See Results for more details.

development of virus-induced CPE and also expression of GFP (cells transfected with pAMPV/CO-GFP plasmid). After one additional passage, the supernatant was harvested and clarified for further purification of the recombinant viruses.

For studies involving virus recovery using heterologous support plasmids, transfections were performed using pAMPV/CO-GFP and pHMPV-GFP (3) and various combinations of the support plasmids (Table 2). Virus recovery was monitored by fluorescent microscopy, and at 72 h posttransfection, the cells were scraped into the medium and the total mixture was harvested and flash frozen for virus titration by plaque assay.

Antibody staining of plaques. The recovered recombinant viruses were grown in Vero cells maintained in Eagle's minimal essential medium supplemented with 2% fetal bovine serum and 0.8% methyl cellulose (Sigma). After incubation for 4 days at 37°C, the overlay was removed and the monolayer was fixed with 4% paraformaldehyde solution. The cells were then permeabilized with 1:1 acetone in phosphate-buffered saline at −20°C for 2 min. The plaques were then incubated with rabbit polyclonal antisera (1:1,000 dilution) raised against a polypeptide (KDNSGPIPQNQRPSS, amino acids 140 to 154 on the N ORF) of the predicted N protein of AMPV/CO, followed by incubation with goat anti-rabbit immunoglobulin G tagged with horseradish peroxidase (KPL, Maryland). The plaques were visualized using light microscopy after being stained with diaminobenzidine substrate (Sigma).

Plaque assay. Plaque assays to measure the virus titers were performed on 24-well plates containing confluent Vero cells. The monolayer was incubated with 10-fold serial virus dilutions for 1 h at 37°C. After virus adsorption, the inoculum was removed and replaced with 1 ml of Opti-MEM containing 2% fetal bovine serum and 0.8% methyl cellulose (Sigma), and the cells were incubated at 37°C. For rHMPV-GFP, exogenous trypsin (Fisher Scientific) was included in the methyl cellulose overlay at a concentration of 5 μg/ml. After 4 days postinfection, the methyl cellulose overlays were removed and the cells were fixed with 80% methanol. The fixed monolayer was then blocked with 5% (wt/vol) nonfat dry milk in phosphate-buffered saline, and the cells were incubated with rabbit polyclonal antipeptide antibody (1:1,000 dilution) raised against the AMPV/CO N protein. The cells were then incubated with horseradish peroxidase-labeled goat anti-rabbit antibodies (KPL, Maryland). Viral plaques were counted following incubation with diaminobenzidine substrate chromogen (Sigma) to determine virus titers. For the GFP-expressing viruses, the number of positive foci was counted under fluorescent microscopy and was also confirmed with the number obtained following an immunostaining reaction.

Virus growth. Multiple-step growth characteristics of the recombinant viruses were compared with those of the parental virus. Briefly, Vero cell monolayers grown in six-well plates were infected with 0.01 multiplicity of infection (MOI) of the parental, rAMPV/CO, and rAMPV/CO-GFP viruses. Supernatants (0.2 ml) were collected at the indicated time points postinfection and replaced by an equivalent volume of fresh medium. The collected samples were flash frozen, stored at −70°C, and titrated later in parallel by plaque assay. Each growth curve is based on the average of virus titers calculated from two infected monolayers.

RESULTS

Construction of a plasmid encoding the full-length AMPV/CO genome. A cDNA clone encoding the complete 14,150-nt antigenome of AMPV-C strain Colorado was constructed by sequential cloning of five individual cDNA fragments into the low-copy-number plasmid pBR 322, as shown in Fig. 1. Five overlapping cDNA fragments were generated: fragment 1 contained the putative N, P, and M genes, flanked by a T7 RNA polymerase promoter at the upstream end and an XhoI site at the downstream end. The XhoI site was created by 3-nt substitutions in the putative noncoding region following the termination codon of the M gene and served as a genetic marker to distinguish between the recombinant and the wild-type parental virus. Fragment 2 contained the putative F, M2, and SH genes and was flanked by the XhoI site at the upstream end and a PvuI site at the downstream end. The PvuI site was created by a 1-nt substitution in the putative SH-G intergenic region and also served as a genetic marker to distinguish between the recombinant and the wild-type parental virus. Fragment 3 contained the putative G gene and was bordered on the upstream end by the PvuI site and on the downstream end by an MluI site that was created by 3-nt substitutions in the putative noncoding region following the termination codon of the G gene. Fragment 4 contained the upstream sequences of the putative L gene and was bordered on the upstream end by the MluI site and on the downstream end by a naturally occurring AgeI site. Fragment 5 contained the remaining sequences of the putative L gene and the trailer sequence, flanked by the AgeI site at the upstream end and part of the hepatitis delta virus ribozyme sequence ending in an RsrII site at the downstream end. The RsrII site is a naturally occurring restriction site in the hepatitis delta virus ribozyme. The transcription plasmid vector pBR322 supplied the remaining part of the hepatitis delta virus ribozyme, followed by a T7 RNA polymerase terminator sequence. The full-length plasmid was named pAMPV/CO and was sequenced in its entirety. Sequence analysis revealed that pAMPV/CO was a faithful copy of the 14,150-nt AMPV/CO genome except for the artificially introduced genetic markers at the XhoI, PvuI, and MluI sites.

Construction of full-length plasmid encoding GFP. In order to assist in the recovery and identification of the cDNA-derived virus, the full-length plasmid pAMPV/CO was modified such that it contained a transcription cassette encoding GFP at the MluI site created between the G and L genes. In addition to providing a means to monitor the recovery of rAMPV, this would also help in studying the potential of AMPV to serve as a viral vector. The procedure of cloning the GFP transcription cassette into the full-length plasmid pAMPV/CO is shown in Fig. 2. The foreign gene cassette was flanked by the gene start signal of the N gene and the gene end signal of the G gene to enable proper transcription. The resulting plasmid, pAMPV/CO-GFP, was sequenced at the flanking sites of the MluI cloning site to confirm the insertion of the foreign cassette.

Recovery of infectious recombinant viruses. The antigenome plasmids pAMPV/CO and pAMPV/CO-GFP, along with a panel of the support plasmids encoding the N, P, M2-1, and L

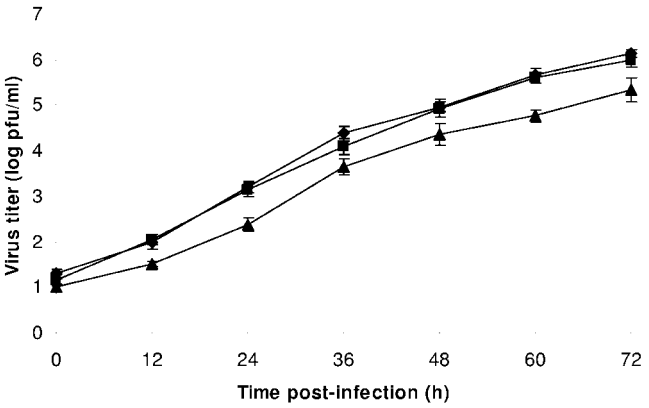

FIG. 3. Replication kinetics of wild-type AMPV/CO, rAMPV/CO, and rAMPV/CO-GFP. Vero cells were infected at a multiplicity of infection of 0.01 with wild-type AMPV/CO (◆), rAMPV/CO (■), or rAMPV/CO-GFP (▲). Supernatants (0.2 ml out of a total volume of 3 ml per well) were taken at the indicated time points postinoculation and replaced by an equivalent amount of fresh medium. The samples were flash frozen and analyzed later for virus titers by plaque assay and immunostaining. Each time point was represented by two wells, and each titration was performed in duplicate. The mean virus titers are shown. Bars indicate standard deviations.

proteins, were transfected into BSR T7/5 cells that stably express the T7 RNA polymerase. In a parallel transfection, plasmid encoding the L protein was excluded to serve as a negative control. In the case of pAMPV/CO-GFP, the transfected cells were examined by fluorescent microscopy on successive days after transfection. Green fluorescent cells were visualized by the day after transfection. Initially, they appeared as scattered isolated cells, and subsequently, they formed foci of more cells that later on developed into well-formed syncytia, similar to the CPE induced by AMPV/CO. When the transfection mixture was transferred to fresh Vero cells, green fluorescent cells were visualized after about 24 h postinfection, denoting that the virus was derived entirely from the cDNA and not a laboratory contamination of AMPV/CO. Recovery of infectious rAMPV/CO-GFP was also possible without the inclusion of M2-1 protein in the transfection mixture (data not shown). DNA sequencing of the RT-PCR products derived from the recovered viruses denoted the presence of the artificially introduced restriction markers, while they were absent in the biologically derived AMPV/CO (data not shown). The recovered recombinant viruses will henceforth be known as rAMPV/CO and rAMPV/CO-GFP.

Multiple-step growth cycle replication of rAMPV/CO, rAMPV/CO-GFP, and wild-type AMPV/CO was evaluated following inoculation of Vero cells at an MOI of 0.01 (Fig. 3). Both the kinetics and magnitude of replication of rAMPV/CO were similar to those of the wild-type virus, indicating that rAMPV/CO was fully competent for multicycle growth in vitro. However, the kinetics and magnitude of replication of rAMPV/CO-GFP were slightly lower than those of the wild-type AMPV/CO as well as those of rAMPV/CO. Virus titers of rAMPV/CO-GFP in the supernatants collected at 72 h postinoculation were around $10^{5.3}$ PFU/ml, while the titers of the other two viruses tested were around $10^{6.0}$ PFU/ml (Fig. 3). Monitoring of Vero cells by fluorescence microscopy following infection with rAMPV/CO-GFP also showed that GFP was stably expressed for at least five serial passages.

The wild-type AMPV/CO, rAMPV/CO, and rAMPV/CO-GFP viruses induced CPE, consisting of large syncytia, in cultured Vero cells (Fig. 4A). The syncytia induced by the three viruses were similar and were indistinguishable. Furthermore, the recovered rAMPV/CO and rAMPV/CO-GFP were compared with the wild-type AMPV/CO for their antigenic characteristics in an immunoperoxidase plaque-staining reaction (Fig. 4B). We observed that the plaques induced by all three viruses were similar in size and shape. These results indicated that the recovered recombinant viruses exhibited growth properties similar to those of the wild-type virus in tissue culture and that the insertion of a foreign gene does not drastically affect the in vitro replication characteristics of AMPV/CO.

Rescue of GFP-expressing viruses by using heterologous support plasmids. In order to examine the functional relationship between AMPV and HMPV polymerase complex proteins, we recovered GFP-expressing AMPV and HMPV by using the support plasmids of either virus. Transfection reactions (as described in Materials and Methods) were performed to recover rAMPV/CO-GFP and rHMPV-GFP, using heterologous support plasmids in various combinations (Table 2). The transfected cells were observed for the appearance of green fluorescent cells, and at 72 h posttransfection, the entire cell lysate was collected and flash frozen. The collected samples were assayed in parallel in Vero cells for virus titers. Each titration was performed in duplicate, and the virus recovery was calculated as the percentage of GFP-expressing virus (rAMPV/CO-GFP or rHMPV-GFP) rescued with its own homologous support plasmids. The results showed that both for AMPV and for HMPV, recovery efficiency was best when the complete set of homologous support plasmids was used. The recovery efficiencies of both recombinants were reduced to about half by using the complete set of four support plasmids from the heterologous virus (Table 2). By using any combination of N, P, M2-1, and L of HMPV and AMPV origins, virus recovery was further reduced, indicating that species-specific protein-protein interactions are more important for a functional ribonucleoprotein complex than protein-RNA interactions.

DISCUSSION

In this study, we report for the first time the recovery of infectious rAMPV/CO and infectious rAMPV/CO-GFP entirely from cloned cDNA. The complete genome data available for AMPV/CO (17) was used to generate a reverse genetics system for this emerging turkey pathogen. The recombinant virus representing the full-length AMPV/CO (rAMPV/CO), developed in this study, showed growth characteristics similar to those of the biologically derived parental virus in tissue culture. Hence, the rescue of a wild-type-like rAMPV/CO entirely from cloned cDNA confirmed that the currently available nucleotide sequences of AMPV/CO (17) are accurate and functional.

Ever since its identification in the United States, AMPV-C has become a major problem for turkey farmers. Currently, no effective vaccine is available to control AMPV infections in affected birds, thus leading to major economic losses for the U.S. turkey industry. Hence, engineering a safe and effective

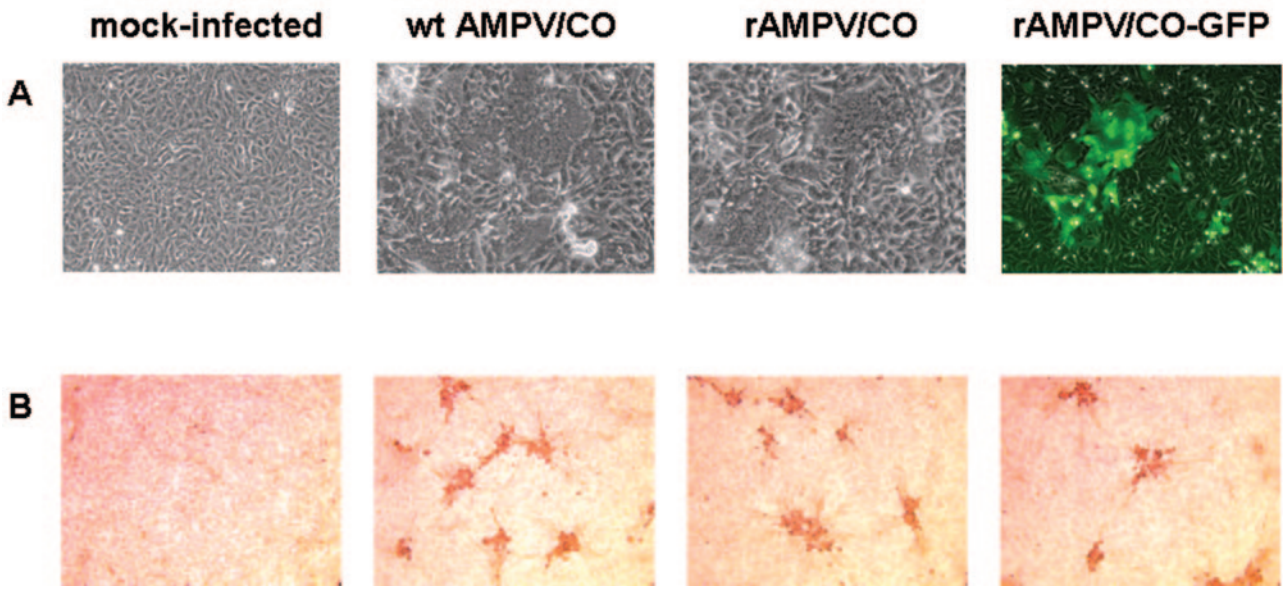

FIG. 4. (A) Cytopathic effect of recombinant AMPV/CO in Vero cells. Vero cells were either infected with wild-type (wt) AMPV/CO, rAMPV/CO, or rAMPV/CO-GFP or mock infected with phosphate-buffered saline and observed for the appearance of CPE characteristic of AMPV. The recovered recombinant viruses induced syncytia indistinguishable from those of wild-type AMPV/CO, at 72 h postinfection and at an MOI of 0.1. (B) Immunostaining of AMPV/CO-infected Vero cells. Vero cells were either infected with 0.1 MOI of wt AMPV/CO, rAMPV/CO, or rAMPV/CO-GFP or mock infected with phosphate-buffered saline, and the infected monolayer was grown in 2% Eagle's minimal essential medium and 0.8% methyl cellulose overlay. After 72 h of infection, the overlay was removed and the monolayers were subjected to immunoperoxidase staining. An antipeptide antibody raised against the N protein of AMPV/CO was utilized for the staining. The recovered recombinant virus-induced plaques were indistinguishable in morphology as well as staining characteristics from those induced by wild-type AMPV/CO.

live recombinant vaccine for the U.S. subgroup of AMPV becomes imperative. Unfortunately, the recently developed reverse genetic system for AMPV-A (24) cannot be used to generate live recombinant vaccines to control AMPV infections in the United States for two main reasons: (i) AMPV-A is very different both genetically and antigenically from subgroup C and hence would not be effective against the latter, and (ii) an AMPV-A-based vaccine in the United States would mean the introduction of a new subgroup of AMPV into the United States, which is prohibited. Hence, our newly developed reverse genetics system for AMPV subgroup C will be a very effective tool towards the development of a live attenuated vaccine against AMPV infections. In addition, this reverse genetics system will also be helpful in understanding the role of individual metapneumovirus genes in pathogenesis in its natural host, turkey.

An important application of the reverse genetics system is to engineer paramyxoviruses to express additional foreign genes. Studies over the last many years have indicated that the genomes of paramyxoviruses are very elastic and that they can be manipulated to stably express foreign proteins to very high levels. Recently, the recovery of recombinant HMPVs expressing GFP and multiple extra genes indicated that the HMPV genome can accommodate an additional 30% of its original genome size (3). In the present study, the reverse genetics system developed by us was used to generate a recombinant AMPV/CO that expresses GFP. A GFP coding sequence was inserted as a transcription cassette in the G/L intergenic region of full-length AMPV/CO cDNA. The recovered rAMPV/CO-GFP virus showed growth properties in tissue culture similar to those of the parental virus but yielded viral titers 1 log lower than those of the parental virus. We also observed that rAMPV/CO stably expressed GFP for at least five serial passages in Vero cells. These results indicated that AMPV/CO can be genetically manipulated through reverse genetics to stably express foreign proteins to relatively high levels and thus has a great potential to serve as a viral vector not only for avian species but also for humans. Furthermore, the ability to express a foreign protein from an artificially inserted transcription cassette also confirmed the identification of the transcription signal sequences of AMPV/CO. This rAMPV/CO-GFP will also be helpful in future studies involving virus tropism and pathogenesis.

HMPV, the mammalian counterpart of AMPV, is a recently discovered virus causing respiratory illnesses in young, adult, and immunocompromised individuals (5, 23, 36, 38). Currently, AMPV and HMPV are the only members of the genus *Metapneumovirus*, and these viruses possess identical genome structures. AMPV subgroup C and HMPV possess very high levels of nucleotide and amino acid identities (16–18, 34, 35, 42). The amino acid identities between the N, P, M2-1, and L proteins of AMPV/CO and HMPV are 88, 67, 85, and 80%, respectively (17). These two viruses also share a high degree of nucleotide similarity in the transcription start and stop signals and in the genomic leader and trailer sequences. The leader and trailer regions of AMPV/CO showed greater similarity to corresponding regions of the HMPVs than to those of AMPV-A (Table 3). The 3′ leader regions of all known metapneumoviruses are identical for 18 of the first 20 positions, while the 5′ trailer regions are identical for 20 of the first 22

TABLE 3. Genomic termini of metapneumoviruses

Region and virus	Length (nt)	Sequence (genomic RNA sense)[a]	Identity (%)
Leader (3′-5′)			
AMPV/CO	40	UGCUCUUUUUUUGCGUAUAUUCUGUUGAAGGUUUGUUUUG-	
AMPV-A	41	.....AG...GUCCA.GAUC..U..A.UA	65
HMPV-NL-00-1	41	...G.....C.....UAA.CU.....U...AUA	75
HMPV-NL/1/99	40	...G.....C.....UAA..U.....-	85
CAN97-83	41	...G.....C.....UAA..C..U....U...GUA	75
CAN98-75	40	...G.....C.....UAA..U.....-	85
Trailer (5′-3′)			
AMPV/CO	39	ACGGCAAAAAAACCGUAUUCAUCCAAUUUUAGUUCCUCA-	72
AMPV-A	40	...AG.....A.A....UAGCUU.U.G	85
HMPV-NL-00-1	40	.....A...U.....A..A..U..U.U	85
HMPV-NL/1/99	40	.....A...U.....C.A..U..U.U	85
CAN97-83	40	.....A...U.....A..A..U..U.U	85
CAN98-75	40	.....A...U.....C.A..U..U.U	

[a] Perfectly conserved nucleotides relative to AMPV/CO are indicated by dots, and gaps are represented by dashes. The genomic terminus sequences were obtained from the GenBank database: HMPV-NL-00-1, AF371337; HMPV-NL/1/99, AY525843; CAN97-83, AY297749; CAN98-75, AY297748; AMPV-A strain LAH A, AY640317. The terminal 40 nt of the trailer regions were utilized only for comparison.

positions. This high degree of conservation suggests that the genomic and antigenomic promoters of metapneumoviruses probably lie within the terminal 20 nt of the genome. Given these similarities, it is possible that the polymerase complex proteins of AMPV/CO support the recovery of HMPV and vice versa. It will be interesting to study whether AMPV-A support plasmids could also recover infectious AMPV/CO or HMPV.

It is generally believed that an infectious virus system is more sensitive than a minigenome system for studying "rescue" using heterologous support proteins (33). We have utilized the recently developed GFP-expressing infectious clone systems of AMPV/CO and HMPV (3) to examine the functional relationship between these two viruses. From our study, we observed that homologous sets of plasmids (i.e., all plasmids from the same virus) were able to recover infectious viruses with better efficiency than heterologous sets. Similar results were also obtained for morbillivirus minigenome rescues by others (6). A reduction in the efficiency of virus recovery with heterologous protein combinations probably denotes that protein-protein interactions are more critical than protein-RNA interactions (33). The results from our study that the polymerase complex of either virus can be cross-recognized denote that all *cis*-acting elements of AMPV-C and HMPV are probably functionally conserved. Numerous previous studies have also shown that the polymerase complex proteins of a virus can support the replication of another virus within the same genus (7, 19, 27, 33, 41). Our findings that the support plasmids of AMPV can successfully rescue HMPV and vice versa establish, for the first time, a functional resemblance between these two metapneumoviruses.

This newly developed virus recovery system will be very helpful in studying basic molecular biology of metapneumoviruses in general and in developing attenuated live recombinant vaccines to control this emerging poultry pathogen. Several live attenuated and chimeric recombinant viruses have been engineered through reverse genetics techniques (9, 12, 15, 30). Using similar techniques, potential vaccine candidates can also be generated to control AMPV/CO. For instance, gene-deleted recombinant HMPVs lacking the SH and/or G gene (4) and the M2-2 ORF (8) were found to be attenuated. Similar versions of AMPV/CO can also be generated and used as vaccine candidates. Recombinant AMPV/CO lacking the G glycoprotein can be expected to be a promising vaccine virus, because it will replicate efficiently but not spread to multiple tissues and hence be attenuated. Chimeric live attenuated HMPVs in which the N or P gene was replaced with that of AMPV/CO were recently generated (28). Similarly, chimeric AMPV/COs with the N or P gene of HMPV can be generated and examined for their vaccine potential. Using this system, AMPV/CO can also be engineered to carry foreign proteins of viruses causing diseases in other avian, nonavian, or human species and their potential as a vaccine vector can be further investigated.

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